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Staphylococcus aureus related to bovine mastitis in Switzerland: Clonal diversity, virulence gene profiles and antimicrobial resistance of isolates collected throughout 2017

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Contents

***Staphylococcus aureus* related to bovine mastitis in Switzerland: Clonal diversity, virulence gene profiles and antimicrobial resistance of isolates collected throughout 2017**

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Abstract

Staphylococcus aureus can be associated with bovine mastitis, leading to high financial losses in dairy industry worldwide. In addition, milk products are one of the most common food categories implicated in Staphylococcal Food Poisoning in humans. In this study, we assessed the population structure, phenotypic resistance patterns and virulence and resistance gene profiles of 58 *S. aureus* strains isolated from bovine mastitis milk in Switzerland. DNA microarray was used to test for virulence and resistance genes, while minimum inhibitory concentrations of various antimicrobial agents were determined by microdilution. We determined clonal complexes and performed multilocus sequence typing and *spa* typing. The strains were assigned to seven clonal complexes, 10 sequence types and 11 *spa* types, with CC705 (43%), CC97 (33%), and CC20 (12%) representing the most common lineages and t529 (43%) and t267 (21%) representing the most common *spa* types. Only one isolate was assigned to CC8, which is linked to high within-herd prevalence of mastitis. A total of 14% (n = 8) of strains was classified as resistant to penicillin and one strain each was classified as oxacillin and pirlimycin resistant. One strain assigned to CC20, ST389 and t2094 exhibited resistance to penicillin, oxacillin, and pirlimycin as well as intermediate susceptibility to erythromycin.

Keywords

Staphylococcus aureus; bovine mastitis; minimum inhibitory concentration; population structure

Zusammenfassung

Staphylococcus aureus ist ein Erreger boviner Mastitis, welche weltweit zu hohen wirtschaftlichen Verlusten in der Milchindustrie führt. Milchprodukte gelten ausserdem als eine der häufigsten Lebensmittelkategorien, welche mit *S. aureus* bedingten Intoxikationen in Zusammenhang stehen. In dieser Studie wurden 58 *S. aureus* Stämme, isoliert aus bovinen Mastitis-Milchproben, hinsichtlich ihrer Populationsstruktur, phänotypischer Resistenzmuster sowie Resistenz- und Virulenz-Genprofile untersucht. Ein DNA Microarray wurde benutzt, um Virulenz- und Resistenzgene nachzuweisen, während die minimalen Hemmkonzentrationen mithilfe eines Mikrodilutionstests bestimmt wurden. Eine weitergehende Charakterisierung erfolgte mittels MLST und *spa* Typisierung. Die Stämme konnten sieben klonalen Komplexen, 10 Sequenztypen und 11 *spa* Typen zugeordnet werden. CC705 (43%), CC97 (33%) und CC20 (12%) repräsentierten die häufigsten klonalen Komplexe, t529 (43%) und t267 (21%) die häufigsten *spa* Typen. Nur ein Isolat wurde dem CC8 zugeordnet, welcher mit einer hohen Herdenprävalenz für Mastitiden verbunden wird. Insgesamt 14% (n = 8) der Stämme wurden als resistent gegen Penicillin und je ein Stamm resistent gegen Oxacillin und Pirlimycin klassifiziert. Ein Stamm, zugeordnet zu CC20, ST389 und t2094 wies Resistenzen gegen Penicillin, Oxacillin und Pirlimycin auf.

Schlüsselwörter:

Staphylococcus aureus; bovine Mastitis; minimale Hemmkonzentrationen; Populationsstruktur

Introduction

The dairy industry suffers from considerable economic losses due to staphylococcal mastitis in cattle (Wells et al., 1998), with the prevalence of udder infections being closely linked to milking hygiene, as well as udder and leg hygiene (Schreiner and Ruegg, 2003; Neave et al., 1969). Intramammary infections caused by *Staph. aureus* are difficult to cure and are particularly challenging as they are prone to chronicity and resurgence (Peton and Loir, 2014). Though antibiotic treatment is widely used to fight bovine mastitis, its merits are controversially discussed. Use of antimicrobial agents is not only economically questionable and favors the development of antibiotic resistance, but it is also unsuitable to address the issue of intracellular persistence of the organism (Fluit, 2012; Saini et al., 2012; Steeneveld et al., 2011). Therefore, increased efforts are now focused on the development of vaccines. Recent studies postulate extended characterization of the genetic background of bovine mastitis isolates to enable identification of proteins crucial for colonization and infection that could serve as biomarkers in the identification of vaccine targets (Fluit, 2012; Klein et al., 2012). It has been argued that knowledge of local epidemiology is also essential for antimicrobial treatment choices in the absence of susceptibility data (Sakwinska et al., 2011b), as closely related strains frequently share resistance gene patterns. For instance previous publications indicate that isolates of CC705, CC479, and CC20 are only very rarely classified as resistant to antimicrobial agents used to treat bovine mastitis (Sakwinska et al., 2011b; Moser et al., 2013). Further data are therefore needed to either corroborate or relativize such findings.

Bovine intramammary *Staph. aureus* infections are also of relevance in the context of foodborne intoxications in humans. Ingestion of food containing staphylococcal enterotoxins leads to Staphylococcal Food Poisoning (SFP) characterized by violent vomiting, diarrhea, and prostration (Fetsch and Johler, 2018). While food handlers contaminating food with *Staph. aureus* are considered the most common source of SFP, outbreaks have also been linked to consumption of raw milk or raw milk cheese originating from dairy animals suffering of mastitis (Johler et al., 2015; Giezendanner et al., 2009).

The objective of this study was to assess the population structure, phenotypic and genotypic resistance patterns and virulence and resistance gene profiles of *Staph. aureus* isolates from bovine mastitis milk in Switzerland in order to provide data needed to improve treatment of bovine mastitis.

Materials and methods

Bacterial Isolation and Species Identification

Samples were submitted by the ambulatory veterinary service of the farm animal clinic of University of Zurich to the routine diagnostic laboratory of the Institute for Food Safety and Hygiene, University of Zurich (January 2017 to January 2018). A total of 58 milk samples originating from 58 different cows and 38 farms, with the vast majority of farms being located in the canton of Zurich (30 out of 38 farms) was included in the study. Information on the geographical origin of the samples, the farm and (if available) on California Mastitis Test (CMT) results and the type of mastitis diagnosed are provided in Supplement 1 (only in the online version of the paper).
Bacteriological examinations of the milk samples were performed according to standard procedures (National Mastitis Council, 1999). Species identification was performed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) spectrometry (Bruker BioSpin AG, Fällanden, Switzerland) according to manufacturer's instructions.

DNA Extraction

DNA isolation was performed following the protocol suggested by the DNA microarray provider (StaphyType ArrayStrips, Alere/ Abbott Laboratories, Jena, Germany) using extraction kits supplied by QIAGEN (Hilden, Germany). A Nanodrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to determine the concentration of nucleic acids.

Multi Locus Sequence Typing (MLST)

MLST was performed with all strains using the GoTaq PCR system (Promega AG, Dübendorf, Switzerland) and primers and cycling conditions previously described (Enright et al., 2000). Briefly, target regions of *arcC, aroE, glpF, gmk, pta, tpi*, and *yqiL* were amplified, PCR products were subsequently purified and sequencing was outsourced (Microsynth, Balgach, Switzerland). The allelic profile of each strain was identified and assigned to the respective sequence type (ST) using the PubMLST database (https://pubmlst.org).

spa Typing

The polymorphic X region of the *spa* gene was determined for all strains using spa-1113f (5'-TAA AGA CGA TCC TTC GGT GAG C-3') and spa-1514r primers (5'-CAG CAG TAG TGC CGT TTG CTT-3') (Aires-de-Sousa et al., 2006) and the GoTaq PCR system (Promega AG) as previously described (Wattinger et al., 2012a). PCR amplicons were purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and sequencing was outsourced (Microsynth, Balgach, Switzerland). Subsequently, *spa* types were determined using the *spa*-server (http://spa.ridom.de/) (Harmsen et al., 2003).

DNA Microarray based Genotyping, Clonal Complex Prediction, and SplitsTree Analysis

The presence/ absence of >300 genes and allelic variants was assessed for all strains using StaphyType ArrayStrips (Alere/ Abbott Laboratories, Jena, Germany) according to the manufacturer's instructions. Multiplex linear DNA amplification and microarray hybridization enables identification of species markers, genes conferring resistance to antimicrobial agents, and virulence determinants such as genes encoding enterotoxins, leukocidins, hemolysins, and microbial surface components recognizing adhesive matrix molecules. The microarray also predicts assignment of strains to clonal complexes. DNA microarray profiles were converted to sequence-like strings as previously described (Wattinger et al., 2012a) to enable visualization using SplitsTree, a software package

designed to compute unrooted phylogenetic networks from molecular sequence data (Huson and Bryant, 2006).

Susceptibility Testing

Minimum inhibitory concentrations (MIC) of various antimicrobial agents were determined for all 58 isolates by microdilution using the MICRONAUT-S Mastitis 3 system (MERLIN, Bornheim-Hersel, Germany) in accordance with the manufacturer's instructions. The test system includes the following antimicrobial agents: penicillin (0.125-8 µg/ml), ampicillin (4-16 µg/ml), cefazolin (4-32 µg/ml), cefoperazone (2-16 µg/ml), cefquinome (1-8 µg/ml), oxacillin (1-4 µg/ml), pirlimycin (1-4 µg/ml), erythromycin (0.125-4 µg/ml), marbofloxacin (0.25-4 µg/ml), amoxicillin/ clavulanic acid (from 4 µg/ml amoxicillin and 2 µg/ml clavulanic acid to 32 µg/ml amoxicillin and 16 µg/ml clavulanic acid), and kanamycin-cefalexin (from 4 µg/ml kanamycin and 0.4 µg/ml cefalexin to 32 µg/ml kanamycin and 3.2 µg/ml cefalexin).

Results

Species confirmation of all 58 isolates identified as *Staph. aureus* by MALDI-TOF MS was achieved using DNA microarray species markers. To avoid bias, the collection was screened for isolates representing the same strain by comparison of all features tested including microarray profiles and resistance patterns, and all isolates were found to represent unique strains. The strains were assigned to 7 clonal complexes (CC), 10 sequence types and 11 *spa* types. The most common clonal lineages were CC705 (former CC151; 43%), CC97 (33%), and CC20 (12%). Strains were only rarely assigned to CC1 (3%), CC479 (5%), CC8 (2%), and CC15 (2%). The most common *spa* types were t529 (43%) and t267 (21%), followed by t524 (9%) and t2094 (7%). An overview of the typing data is provided as Table 2 and comprehensive information can be found in Supplement 1 (only in the online version of the paper).

For 10 of the farms included in the study, more than 1 isolate was available and 3 farms had known *Staph. aureus* mastitis problems at herd level (see Table 3). An overview of the clonal complexes, sequence types, and *spa* types associated with these isolates is presented in Table 3. A SplitsTree was used to construct an unrooted phylogenetic network depicting relatedness of all isolates based on similarity of DNA hybridization profiles (see Figure 1). All strains assigned to the same clonal complex formed clusters in the SplitsTree. Strains that originated from the same herd were color coded.

Virulence and resistance gene profiles of all strains were determined by DNA microarray. While none of the strains carried *mecA/mecC* associated with methicillin-resistant *Staph. aureus*, NK_7 (CC8, ST8, t2953) harbored cassette chromosome recombinase genes *crrA* and *crrB*. In addition, we detected the fosfomycin resistance gene *fosB*, which encodes a metallothiol transferase. The *tst* gene was detected in seven out of 25 strains assigned to CC705. None of the strains was positive for the genes encoding exfoliative toxins associated with staphylococcal scalded skin syndrome or carried genes coding for epidermal cell differentiation inhibitors.

A microdilution approach was chosen to determine the susceptibility of all 58 strains to different antimicrobial agents. An overview of resistance phenotypes and MIC_{50}/MIC_{90} values, indicating the MIC at which ≥ 50% and ≥ 90% of the strains tested in our study would be inhibited, is presented in Table 1 and Figure 2. Comprehensive data on MIC distribution is available as Supplement 2 (only in the online version of the paper). For eight of the antimicrobial agents tested, no breakpoints were available. For penicillin, oxacillin, erythromycin, and kanamycin/cefalexin, human clinical breakpoints were used. A total of 14% (n = 8) of the strains was classified as resistant to penicillin and one strain each was classified as oxacillin and pirlimycin resistant. Growth of all strains was inhibited by the lowest combination of kanamycin/ cefalexin concentrations tested (4 µg/ml kanamycin and 0.4 µg/ml cefalexin). No clinical breakpoints were available for ampicillin, cefazolin, cefoperazone, cefquinome, marbofloxacin. Also, no clinical breakpoints were available for amoxicillin/ clavulanic acid. With one exception, all isolates yielded MICs of ≤ 4 µg/ml and ≤ 2 µg/ml, which represented the lowest concentration tested. Only for one strain (NK_18), a higher MIC was determined for this combination of antimicrobial agents (32 µg/ml amoxicillin and 16 µg/ml clavulanic acid). NK_18 exhibited resistance to penicillin, oxacillin, and pirlimycin as well as intermediate susceptibility to erythromycin and high MICs for several antimicrobial agents, for which no breakpoints were available: cefazolin MIC > 32, erythromycin MIC > 0.5, amoxicillin/ clavulanic acid MIC > 16/8, and marbofloxacin MIC > 0.25. NK_18 was assigned to CC20, ST389, and t2094 and originated from a farm, for which only one milk sample was submitted (F14). The milk sample was collected from a cow with subclinical mastitis and a positive California Mastitis Test in mid-lactation period. We

included three farms in this study, for which herd problems with *Staph. aureus* mastitis are known. For one of these farms (F11), a *Staph. aureus* of the same clonal complex, sequence type and *spa* type (CC20, ST389, t2094) as NK_18 was detected that was susceptible to all tested antimicrobial agents.

Discussion

None of the strains in this study was detected more than once. In total, 7 CCs were predicted by DNA microarray and further differentiated by MLST and *spa* typing into 10 sequence types and 11 *spa* types. While *spa* typing yielded overall higher resolution than MLST, the most common bovine *spa* type t529 could be subdivided into two sequence types (ST504 and ST151). The most prevalent clonal lineages in this study were CC705 (former CC151) and CC97. These clonal complexes are frequently detected in *Staph. aureus* collected from cases of bovine intramammary infections in Switzerland and worldwide (Fitzgerald, 2012; Moser et al., 2013; Stalder et al., 2014; Johler et al., 2011; Sakwinska et al., 2011a; Boss et al., 2016). CC705 is the major clone detected among *Staph. aureus* associated with bovine mastitis and was reported to occur exclusively in the bovine host (Sakwinska et al., 2011a; Boss et al., 2016; Fitzgerald, 2012). In contrast, CC97 strains, which were assigned to the *spa* types t267 and t359 also detected in this study, were linked to infections with methicillin resistant *Staph.* aureus (MRSA) in humans (Ellington et al., 2008). The MRSA isolates from wound infection and cellulitis cases in humans carried enterotoxin genes *sed* and *sej* (Ellington et al., 2008), which were missing in methicillin susceptible *Staph. aureus* isolates from cattle included in this study. CC20 is not exclusively linked to mastitis in cattle (Hasman et al., 2010). This clonal lineage causes also infections in humans (Wattinger et al., 2012a; Luedicke et al., 2010) and was reported to be significantly associated with infective endocarditis (Nethercott et al., 2013), as well as with cases of Staphylococcal Food Poisoning (Suzuki et al., 2014). Interestingly, only one of the bovine mastitis isolates was assigned to CC8, which was linked to *Staph. aureus* of genotype B associated with high within-herd prevalence of mastitis in cattle (Graber et al., 2009). CC8 is also known as pandemic MRSA lineage, with numerous community and hospital associated MRSA strains originating from this clonal complex (Monecke et al., 2011). The penicillin resistant CC8 isolate in this study originated from a cow with chronic mastitis that was part of a herd, for which another milk sample harboring a CC705 strain from a cow with subclinical mastitis was submitted. In total, seven of the ten herds, for which multiple cows were sampled, were affected by more than one *Staph. aureus* strain. MRSA strains harbor one of several staphylococcal cassette chromosome *mec* (SCC*mec*) variants. These SCC*mec* are mobile genetic elements that carry *mecA/mecC* coding for penicillin binding protein 2a (PBP2a), a transpeptidase conferring resistance to all beta lactam antibiotics except ceftobipirole and ceftaroline. The DNA microarray array used in this study covers not only *mecA* and *mecC*, but also various other SCC-associated markers such as recombinase genes, *fusC* etc. None of the strains in this study carried *mecA* or *mecC*. However, NK_7 (CC8, ST8, t2953) harbored staphylococcal cassette chromosome recombinase genes *crrA* and *crrB*, which enable excision and integration of SCC*mec* (Katayama et al., 2000; Wang and Archer, 2010). In addition, strain NK_18 (CC20, ST389, t2094) was classified as resistant to oxacillin, although neither *mecA*/*mecC*, nor other SCC-associated genes were detected by DNA microarray. It has recently been shown that the commonly known twelve SCC*mec* types and their variants are only a fraction of the true diversity of SCC*mec* elements (Monecke et al., 2016, 2018). Therefore, a modification of the respective genomic elements or a variant not covered by the microarray used may be present in NK_18.

Overall, phenotypic antimicrobial resistance among the bovine mastitis strains was low, with only 14% (n = 8) of strains classified as resistant to penicillin and one strain each classified as oxacillin and pirlimycin resistant. This is consistent with previous studies (Sakwinska et al., 2011b; Moser et al., 2013) investigating antimicrobial susceptibility in *Staph. aureus* strains collected from bovine mastitis milk in Switzerland. Previous studies in the US, Chile, and Europe classified between 2% and 61% of *Staph. aureus* tested as resistant to penicillin

(Oliver et al., 2012). While no clinical breakpoints are available for the combination of kanamycin/ cefalexin, growth of all strains was inhibited by the lowest combination of kanamycin/ cefalexin concentrations tested (4 µg/ml kanamycin and 0.4 µg/ml cefalexin).
We detected the fosfomycin resistance gene *fosB*, which encodes a metallothiol transferase in CC8, CC15 and CC20. The results are in accordance with a previous paper reporting that *fosB* is specific for certain clonal complexes including CC15 and CC20 (Monecke et al., 2008). In strains of CC705, we also frequently detected the *tst* gene encoding the toxic shock syndrome toxin, a superantigen causing life-threatening disease characterized by fever, desquamation, rash, multisystem failure and shock (Fraser and Proft, 2008). We also detected various enterotoxin genes, including genes coding for major enterotoxins SEC and SED, and the enterotoxin gene cluster (*egc*). Ingestion of staphylococcal enterotoxins preformed in food leads to SFP, resulting in violent vomiting, often accompanied by diarrhea, fever, and prostration (Fetsch and Johler, 2018). While food handlers colonized or infected with *Staph. aureus* represent the most common source of SFP strains (Wattinger et al., 2012b), outbreaks have also been linked to consumption of raw milk or raw milk cheese originating from dairy animals suffering of mastitis (Johler et al., 2015; Giezendanner et al., 2009).
It has been argued that knowledge of local epidemiology is essential for antimicrobial treatment choices in the absence of susceptibility data (Sakwinska et al., 2011b). Previous reports indicate that isolates of CC705, CC479, and CC20 are only very rarely classified as resistant to antimicrobial agents used to treat bovine mastitis (Sakwinska et al., 2011b; Moser et al., 2013). In our study, all CC705 and CC479 strains were susceptible to all antimicrobial agents tested. The same was true for NK_11, a CC20 (ST389/t2094) strain associated with herd problems with *Staph. aureus* mastitis but not for the other CC20 strains, indicating the onset of new resistance patterns. Also, NK_18, the strain exhibiting resistance to most antimicrobial agents was assigned to CC20 (ST389/t2094).
Thus, our findings stress the crucial need for susceptibility testing before selecting antimicrobial agents for treatment of bovine mastitis, even though the local distribution of lineages may be well known. In addition, further veterinary clinical breakpoints are urgently needed to allow for accurate susceptibility classification and to improve therapeutic recommendations.

Tables

Table 1: Resistance phenotypes and MIC_{50} / MIC_{90} values determined for 58 *Staph. aureus* strains isolated from bovine mastitis milk. A comprehensive overview of the distribution of MICs is provided in Supplement 2 (only in the online version of the paper).

Antimicrobial agent	Susceptible (%)	Intermediate (%)	Resistant (%)	MIC_{50}[2]	MIC_{90}[3]	Clinical breakpoints applied
Penicillin	86	-	14	0.125	2	Human clinical breakpoint[1]
Ampicillin	-	-	-	4	4	-
Cefazolin	-	-	-	4	4	-
Cefoperazone	-	-	-	2	2	-
Cefquinome	-	-	-	1	1	-
Oxacillin	98	-	2	1	1	Human clinical breakpoint[1]
Pirlimycin	98	-	2	1	1	Breakpoint for use in cattle
Erythromycin	98	2	-	0.125	0.25	Human clinical breakpoint[1]
Marbofloxacin	-	-	-	0.25	0.5	-
Amoxicillin/ clavulanic acid	-	-	-	4/ 2	4/ 2	-
Kanamycin/ cefalexin	100	-	-	4/ 0.4	4/0.4	Human clinical breakpoint[1]

[1]For many antimicrobial agents tested, breakpoints for use in cattle are missing. Therefore, human breakpoints were used for classification as susceptible/ intermediate/ resistant.
[2] MIC_{50}: concentration of antimicrobial agent that inhibits growth of 50% of all tested strains.
[3] MIC_{90}: concentration of antimicrobial agent that inhibits growth of 90% of all tested strains.

Table 2: Overview of typing and characterization results. Clonal complexes are sorted by frequency of occurrence.

CC	ST	*spa* type	Resistance genes[1]	Superantigens[1]
CC705 (n = 25)	ST504 (n = 22)	t529 (n = 22)	-	*tst, sec, sel, egc*
	ST151 (n = 3)	t529 (n =3)	-	*tst, sec, sel, egc*
CC97 (n = 19)	ST352 (n = 14)	t267 (n = 12)	-	-
		t359 (n = 2)	-	-
	ST71 (n = 5)	t524 (n = 5)	*blaZ/I/R*	-
CC20 (n = 7)	ST389 (n = 6)[2]	t164 (n = 3)	*fosB*	*egc*
		t2094 (n = 4)	*fosB*	*egc*
CC1 (n = 2)	ST1 (n = 2)	t127 (n = 2)	-	*seh*
CC479 (n = 3)	ST479 (n = 2)	t528 (n = 2)	-	*egc*
	ST1380 (n =1)	t2873 (n =1)	-	*egc*
CC8 (n = 1)	ST8 (n =1)	t2953 (n =1)	*crrA/B, blaZ/I/R, fosB*	*sed, sej*
CC15 (n = 1)	ST582 (n =1)	t084 (n =1)	*blaZ/I/R, fosB*	-

[1] The presence of selected resistance and virulence genes determined by DNA microarray is stated. Genes encoding resistance determinants and superantigens were present in at least one of the strains of this CC/ST/*spa* type combination. For a full report of all microarray data for each single strain see Supplement 1 (only in the online version of the paper). None of the isolates harbored mecA/mecC characteristic for MRSA, *pvl* encoding Panton-Valentine leukocidin, or genes encoding exfoliative toxins.

[2] One strain could not be assigned.

Table 3: Overview of clonal complexes (CC), sequence types (ST) and *spa* types for isolates either originating from the same herd or linked to farms with known *Staph. aureus* mastitis problems at herd level.

Herd	**CC**	**ST**	***spa* type**	**Isolates**
F1 (n = 4)	CC705	ST504	t529	NK_01, NK_28, NK_44, NK_48
F3 (n = 2)	CC705	ST504	t529	NK_03
	CC20	ST389	t164	NK_19
F4 (n = 7)	CC97 (n = 5)	ST71	t534	NK_04, NK_13, NK_23, NK_24, NK_54
	CC1 (n = 2)	ST1	t127	NK_12, NK_14
F7 (n =2)	CC8	ST8	t2953	NK_07
	CC705	ST504	t529	NK_56
F9 (n =2)	CC20	ST389	t164	NK_34
	CC705	ST151	t529	NK_09
F10 (n =4)	CC20 (n = 1)	ST389	t164	NK_40
	CC97 (n = 3)	ST352	t267	NK_10, NK_19
			t359	NK_58
F11* (n = 1)	CC20	ST389	t2094	NK_11
F12 (n =3)	CC479 (n = 2)	ST1380	t528	NK_15, NK_42
	CC705 (n = 1)	ST504	t529	NK_51
F18 (n =2)	CC97 (n = 2)	ST352	t267	NK_25, NK_47
F21* (n =2)	CC705 (n = 2)	ST504	t529	NK_29, NK_30
F23* (n =3)	CC705 (n = 2)	ST_504 (n = 1)	t529	NK_32
		ST_151 (n = 1)	t529	NK_36
	CC479 (n = 1)	ST479 (n = 1)	t2873	NK_50

*For three farms (marked by an asterisk), herd level problems with *Staph. aureus* mastitis were known.

Figures

Figure 1. SplitsTree depicting the relatedness of the bovine *Staph. aureus* strains based on similarity of DNA microarray profiles. Proximity in the unrooted phylogenetic network indicates similarity of DNA hybridization profiles and thus relatedness of the respective isolates. All strains assigned to the same clonal complex formed clusters in the SplitsTree. NK_18 (marked by an asterisk), the strain that exhibited resistance to multiple antimicrobial agents including amongst others oxacillin, clustered very closely to other CC20 isolates of both t2094 and t164 that did not exhibit the same resistance profile. Isolates that originated from the same farm are marked by colored circles (farm F1: yellow, farm F3: orange, farm F4: pink, farm F7: light green, farm F9: dark green, farm F10: light blue, farm F11: dark blue, farm F12: grey, farm F18: red, farm F21: purple, farm F23: brown).

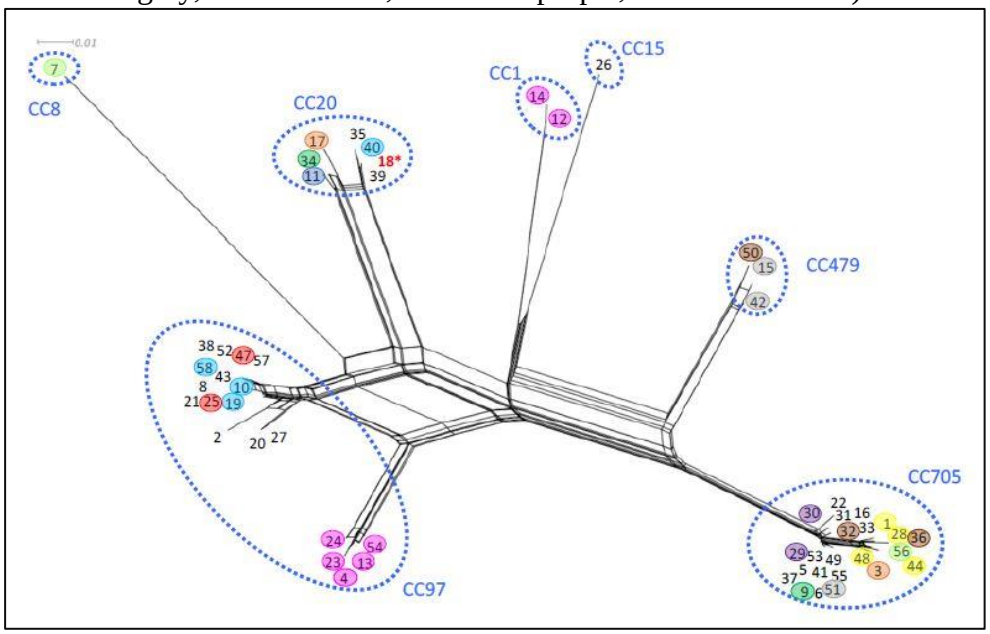

Figure 2. Overview of minimum inhibitory concentrations (**MIC) for the 58 *Staph. aureus* isolates tested.** Numbers indicate the number of strains exhibiting the corresponding MIC value. Light blue areas indicate the tested concentration range on the microdilution plate. Values above this range denote MIC values greater than the highest concentration tested. If available, MIC breakpoints are indicated using vertical lines (red = human breakpoint, blue = bovine breakpoint) separating resistant and susceptible strains, with dotted lines indicating the differentiation between susceptible and intermediate. Breakpoints were obtained from the CLSI guidelines 2017 for human and 2015 for veterinarian breakpoints. MIC_{50} and MIC_{90} represent the concentration of antimicrobial agent inhibiting growth of 50% or 90% of strains, respectively. In the case of kanamycin/ cefalexin, the MIC breakpoint is indicated for kanamycin only (marked by an asterisk).

	minimal inhibitory concentrations of antimicrobial agents in µg/ml										MIC50%	MIC90%
	0.125	0.25	0.5	1	2	4	8	16	32	64		
Penicillin	64	1			2	3	1		2		0.125	2
Ampicillin						71	2				4	4
Cefazolin						72				1	4	4
Cefoperazone					66	7					2	2
Cefquinome				73							1	1
Oxacillin				72			1				1	1
Pirlimycin				72			1				1	1
Erythromycin	47	22	3	1							0.125	0.25
Marbofloxacin		62	11								0.25	0.5
						4/2	8/4	16/8	32/16			
Amoxicillin/ clavulanic acid						72			1		4/2	4/2
						4/0.4	8/0.8	16/1.6	32/3.2			
Kanamycin*/ cefalexin						73					4/0.4	4/0.4

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